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Regulatory Properties of Magnesium-dependent Guanylate Cyclase in *Dictyostelium discoideum* Membranes*

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We have characterized a magnesium-dependent guanylate cyclase in homogenates of *Dictyostelium discoideum* cells. 1) The enzyme shows an up to 4-fold higher cGMP synthesis in the presence of GTP analogues with half-maximal activation at about 1 μM guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) or 100 μM guanosine 5′-(β,γ-imido)triphosphate; little or no stimulation was observed with GTP, guanosine mono- and diphosphates or with adenine nucleotides, with the exception of the ATP analogue adenosine 5′-(β,γ-imido)triphosphate. 2) Both basal and GTPγS-stimulated guanylate cyclase activity were rapidly lost from homogenates as was the ability of GTPγS to stimulate the enzyme after cell lysis. 3) Inclusion of 25 μM GTPγS during cell lysis reduced the K_M for GTP from 340 to 85 μM and increased the V_{max} from 120 to 255 pmol/min•mg protein, as assayed in homogenates 90 s after cell lysis. 4) Besides acting as an activator, GTPγS was also a substrate for the enzyme with a K_M = 120 μM and a V_{max} = 115 pmol/min•mg protein. 5) GTPγS-stimulated, Mg^{2+}-dependent guanylate cyclase was inhibited by submicromolar concentrations of Ca^{2+} ions, and by inositol 1,4,5-trisphosphate in the absence of Ca^{2+} chelators. 6) Guanylate cyclase activity was detected in both supernatant and pellet fractions after 1 min centrifugation at 10,000 × *g*; however, only sedimentable enzyme was stimulated by GTPγS. We suggest that the Mg^{2+}-dependent guanylate cyclase identified represents the enzyme that in intact cells is regulated via cell surface receptors, and we propose that guanine nucleotides are allosteric activators of this enzyme and that Ca^{2+} ions play a role in the maintenance of the enzyme in its basal state.

In the cellular slime mold *Dictyostelium discoideum*, intracellular guanylate cyclase is activated up to 10-fold by chemoattractants that bind to cell surface receptors. Other well studied responses regulated via receptors are the activation of adenylate cyclase, the phosphorylation of chemotactic cAMP receptors, and alterations in the polymerization state of actin (1). Signal transduction in *D. discoideum* shows remarkable similarities with that in higher eukaryotes with respect to the functioning of cell surface receptors, the regulation of adenylate cyclase by GTP-binding proteins, the mechanism of desensitization, and the likely presence of a phosphatidylinositol cycle (2). On the other hand, mechanisms have been proposed to operate in *Dictyostelium* which have no precedent in other organisms. Examples are an involvement of the phosphatidylinositol cycle in regulation of guanylate cyclase (3, 4), and the role that *ras* proteins might have in the desensitization of this enzyme (5). The latter findings have attracted attention, also because limited insight exists into the functioning of receptor-regulated guanylate cyclases in eukaryotes in general (*cf.* Ref. 6).

Despite extensive studies on the activation and desensitization of guanylate cyclase in intact *D. discoideum* cells (see Ref. 1), little is known about the direct regulation of the enzyme. There exists only one report on the modulation of guanylate cyclase activity in cell-free preparations via cell surface receptors: when cells were stimulated with cAMP and rapidly lysed, the enzyme was found to be stimulated 3–6-fold (7). *In vitro*, chemicals like adenosine trisphosphates and glycerol modulate the activity of guanylate cyclase (8–10). However, in all these investigations (7–10) millimolar concentrations of Mn^{2+} ions have been used in the enzyme assays, because with Mg^{2+}-GTP little guanylate cyclase activity was found (9, 10). As the intracellular Mn^{2+} concentrations in *Dictyostelium* are in the micromolar range (9) the relevance of these studies for insight into the regulation of guanylate cyclase may be called into question. Permeabilized cells have been an alternative tool to the study of *Dictyostelium* guanylate cyclase. It was described that the elevation of the cGMP concentration evoked by chemoattractants was mimicked by addition of Ca^{2+} ions or inositol 1,4,5-trisphosphate to permeabilized cells (3, 4). Permeabilized cells, however, offer limited possibilities for manipulation of the enzyme and control of its environment.

We have recently managed to identify a guanylate cyclase in cell-free preparations that is active with Mg^{2+}-GTP (11). This enzyme was observed in homogenates, immediately after cell lysis. In this report we describe some salient features of this enzyme, which is present in membranes, has increased activity in the presence of guanosine triphosphates, and is inhibited by Ca^{2+} ions.

EXPERIMENTAL PROCEDURES

Culture Conditions and Cell Lysis—D. discoideum NC4(H) cells were grown, harvested in the late logarithmic phase with 10 mM Na^+/K^+-phosphate buffer, pH 6.5, and freed from bacteria by centrifugation at 100 × *g* for 4 min (12). Cells were developed until aggregation competence by starvation on non-nutrient agar at 6 °C for 16–20 h; subsequently they were harvested, washed, and resuspended at a

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density of 10^8 cells/ml in lysis buffer (40 mM Hepes[1]/NaOH, 3 mM $MgSO_4$, 1 mM EGTA, 10^{-4} M App(NH)p, pH 7.0, unless otherwise indicated). Homogenization was performed at 0 °C by rapid elution of cell suspensions through polycarbonate filters (pore size 3 μm), by a method developed before for lysis of small cell volumes (100–300 μl) (13, 14). This method resulted in >99% cell lysis, as estimated by phase contrast microscopy.

Guanylate Cyclase Assay—Guanylate cyclase activity was measured at 25 °C using the method briefly described before (11). Standard reaction mixture contained 20 mM Hepes/NaOH, 1.5 mM $MgSO_4$, 0.5 mM EGTA, 5×10^{-5} M App(NH)p, 5 mM dithiothreitol, and 0.3 mM GTP (final concentrations), pH 7.0. For investigation of the enzyme kinetics the GTP or GTPγS concentrations were varied, while keeping the Mg^{2+} concentration at 1.2 mM in excess to the guanine nucleotides by adding extra $MgSO_4$. $MnCl_2$, if added, was present at a concentration of 5 mM. Reactions were started between 40 and 120 s after cell lysis, unless otherwise indicated; samples were routinely taken at 0, 40, and 60 s after initiation of the reaction and quenched in 4 N $HClO_4$. Samples were processed, and cGMP produced was measured in a radioimmunoassay (15, 16). Cyclic GMP synthesis was linear up to about 1.5 mg of protein/ml in the guanylate cyclase assay. Cyclic AMP was measured according to the method of Tovey *et al.* (17).

High Performance Liquid Chromatography Analysis—Reaction products formed during the guanylate cyclase reaction were analyzed by high performance liquid chromatography on Lichrosorb RP-18. Samples from the cyclase reaction in $HClO_4$ were neutralized with $KOH/KHCO_3$ (15) and either treated with phosphodiesterase or directly applied to the high performance liquid chromatography column. Phosphodiesterase treatment was done for 1 h at 22 °C with 0.1 mg/ml beef heart phosphodiesterase in the presence of 4 mM $MgCl_2$; the reaction was arrested by 3 min heating at 99 °C. The high performance liquid chromatography column was eluted with 20% methanol, 1 mM K^+ phosphate, pH 6.5, at 1 ml/min. Fractions were collected, lyophilized, and their cGMP content was measured by radioimmunoassay (15, 16).

Materials—GTP, cGMP, ATP, and dithiothreitol were obtained from Sigma; GTPβS, Gpp(NH)p, GDPγS, APP(NH)p, and beef heart phosphodiesterase were from Boehringer (Mannheim, Federal Republic of Germany); [8-^{3}H]cGMP (15 Ci/mmol) and inositol 1,4,5-trisphosphate were from Amersham International (Amersham, United Kingdom); Lichrosorb 10 RP18 was from Chrompack (Middelburg, The Netherlands), and polycarbonate filters were from Nucleopore (Pleasanton, CA).

RESULTS

Increased Mg^{2+}-dependent Guanylate Cyclase Activity in the Presence of GTPγS

For obtaining Mg^{2+}-dependent guanylate cyclase, cells in a buffer containing Mg^{2+} ions and various additions were lysed by elution through polycarbonate filters, and shortly after lysis homogenates were transferred to a reaction mixture, as described before (11).

When the GTP analogue GTPγS (25 μM) was present during lysis, a marked, about 3-fold higher cGMP production was observed (Table I). It was indeed the activity of a guanylate cyclase that was detected here under cell-free conditions: no cGMP production was obtained with intact cells, and omission of GTP from the reaction mixture resulted in a strong reduction of the cGMP synthesis (Table I), (11). When GTP was replaced by ATP in the reaction mixture a similar reduction of the cGMP production was obtained, while concomitantly the synthesis of cAMP was stimulated strongly (Table I). Although the elimination of GTP from reaction mixtures resulted in reduced cGMP production, this production, especially in the presence of GTPγS, was not zero (Table I). By analysis of high performance chromatography the reaction product synthesized under these conditions was definitely identified as cGMP: reaction products coeluted with authentic cGMP and were degraded by incubation with a cyclic nucleotide phosphodiesterase (data not shown; see also Ref. 11).

TABLE I

Identification of a GTPγS-stimulated, Mg^{2+}-dependent guanylate cyclase in Dictyostelium cell homogenates

Addition/condition[a]		Cyclic nucleotide production	
During lysis	During enzyme reaction	cGMP	cAMP
		pmol/min·mg protein ± S.E. (n)	
Standard	Standard	56 ± 10 (9)	<5
GTPγS	Standard	188 ± 43 (7)	<5
No cell lysis	Standard	<5 (2)	<5
GTPγS, no cell lysis	Standard	<5 (2)	<5
Standard	No GTP	6 ± 1 (3)	
GTPγS	No GTP	28 ± 7 (12)	<5
GTPγS	GTP replaced by ATP	32 (1)	219
Standard	GTP replaced by cGMP	−10 ± 6 (4)	
GTPγS	GTP replaced by cGMP	−18 ± 5 (4)	
Standard	$MnCl_2$	50 ± 15 (7)	
GTPγS	$MnCl_2$	126 ± 22 (4)	

[a] Standard conditions involved cell lysis in 40 mM Hepes\NaOH, 3 mM $MgSO_4$, 1 mM EGTA, 10^{-4} M App(NH)p, pH 7.0 (0 °C). Enzyme reactions were done in 20 mM Hepes\NaOH, 1.5 mM $MgSO_4$, 0.5 mM EGTA, 0.5×10^{-4} M App(NH)p, 0.3 mM GTP, and 5 mM dithiothreitol, pH 7.0, or with modifications as indicated. GTPγS, if indicated, was present at 25 μM, EDTA at 4 mM, $MnCl_2$ at 5 mM, cGMP at 200 or 400 nM, and ATP at 0.3 mM. Cyclic AMP measurements are from one experiment.

The effect of GTPγS on cGMP production resulted from stimulation of a guanylate cyclase and not from inhibition of a phosphodiesterase. This was inferred from measurements of the phosphodiesterase activity under the reaction conditions, which were done by replacing GTP by cGMP. The degradation of cGMP was about 15%/min and was not reduced in the presence of GTPγS (Table I). Furthermore, stimulation of cGMP production by GTPγS was also observed in homogenates from a Streamer F mutant of *D. discoideum* (not shown), which is a strain that lacks cGMP-specific phosphodiesterase (18, 19).

Factors Affecting the Activity of Mg^{2+}-dependent Guanylate Cyclase in the Presence of GTPγS

Time after Cell Lysis—Cell homogenates were prepared in the presence or absence of GTPγS (25 μM) and the activity of Mg^{2+}-dependent guanylate cyclase was measured at different times after cell lysis (Fig. 1). Under both conditions the enzyme activity was rapidly lost. In homogenates prepared in the absence of GTPγS no guanylate cyclase activity was detected at 50 min after cell lysis, whereas in homogenates prepared in the presence of GTPγS the activity was decreased to 10% of the activity directly after cell lysis. The loss of Mg^{2+}-dependent guanylate cyclase in homogenates prepared in the presence of GTPγS showed characteristics of a first order exponential decay, described by a $k_{-1} = 0.115$ min^{-1} ($t_{0.5}$ = 6 min), (Fig. 1, *inset*). The decay of guanylate cyclase activity in homogenates not containing GTPγS appeared to be multiphasic (Fig. 1, *inset*). Shortly after cell lysis it oc-

[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; GDPβS, guanosine 5′-*O*-(2-thio)diphosphate; App(NH)p, adenosine 5′-(β,γ-imido)triphosphate; ATPγS, adenosine 5′-*O*-(3-thio)triphosphate; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

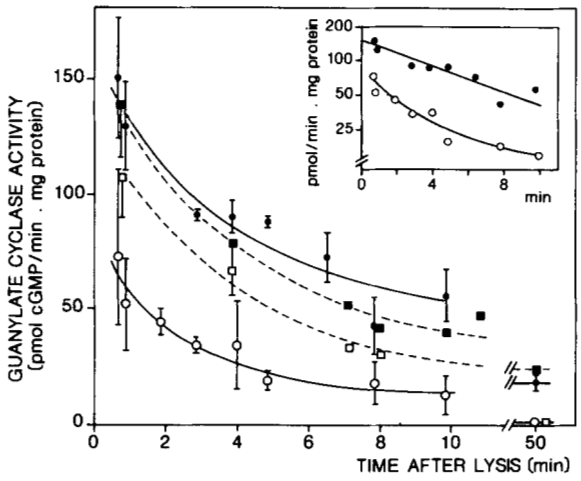

FIG. 1. **Decay of the guanylate cyclase activity in cell homogenates.** Homogenates were prepared in the absence of (○, □) or presence (●, ■) of 25 μM GTPγS at time = 0 min. After various incubation times at 0 °C samples were assayed for guanylate cyclase activity, either under standard conditions (Mg^{2+}/0.3 mM GTP; ○, ●) or in standard reaction medium containing 2 mM GTP and 5 mM $MnCl_2$ (□, ■). The *inset* shows a semilogarithmic plot of the data from reactions not containing Mn^{2+} ions. Data are the means ± S.E. of 2–4 experiments.

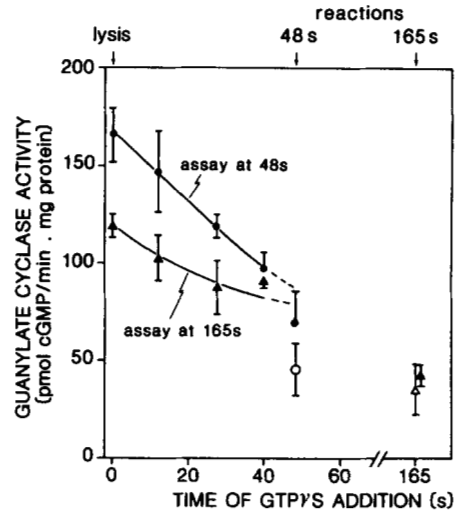

FIG. 2. **Effect of addition of GTPγS after cell lysis on the activity of guanylate cyclase.** *D. discoideum* cells were lysed at time = 0 s and at different times after lysis 25 μM GTPγS was added to homogenates (0 °C) (*filled symbols*). Once 25 μM GTPγS was added prior to lysis (*filled symbols* at $t = 0$ s); in two cases homogenates encountered GTPγS concomitantly with reaction mixture (*filled symbols at 48 and 164 s*). *Open symbols*, guanylate cyclase activities from homogenates that received no GTPγS. For each preparation, the guanylate cyclase activity was assayed at 48 s (○, ●) and 164 s (△, ▲) after cell lysis. Data are the means ± S.E. of measurements from 5 different experiments.

curred with a $t_{0.5} < 3$ min and it approached a $t_{0.5} \approx 6$ min around 8 min after lysis.

About the same decay kinetics were observed when the guanylate cyclase activity in homogenates was measured in the presence of Mn^{2+} ions and 2 mM GTP (Fig. 1). These assay conditions were used as a measure of the total Mg^{2+}- and Mn^{2+}-dependent guanylate cyclase activities, in case that there might exist distinct Mg^{2+}- and Mn^{2+}-dependent enzymes. However, no evidence for distinct Mg^{2+}- and Mn^{2+}-dependent enzymes was found. GTPγS also stimulated guanylate cyclase assayed with Mn^{2+}-GTP (2 mM), but the relative stimulation by GTPγS was lower than as measured with Mg^{2+}-GTP (Fig. 1). This was due to the higher basal guanylate cyclase activity in the former condition as compared to the latter.

GTPγS also stimulated guanylate cyclase when added to homogenates after cell lysis. However, the longer the time elapsed between cell lysis and the addition of 25 μM GTPγS, the lower the stimulation (Fig. 2). Thus, in addition to the instability of the catalytic activity of the enzyme as demonstrated in Fig. 1, the potential of the enzyme to be stimulated by GTPγS fades after cell lysis ($t_{0.5}$ = 30–60 s) (Fig. 2).

Ca^{2+} Ions and Inositol Trisphosphate—Ca^{2+} ions and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) deserve special attention as potential regulators of *Dictyostelium* guanylate cyclase, as it was reported that these compounds induce a cGMP response in saponin-permeabilized *Dictyostelium* cells (3, 4). The effect of Ca^{2+} ions was investigated by varying the free Ca^{2+} concentration in homogenates using Ca^{2+}-EGTA buffers (20). Both basal and GTPγS-stimulated, Mg^{2+}-dependent guanylate cyclase were strongly inhibited by Ca^{2+} ions (Fig. 3). Half-maximal inhibition of both forms of guanylate cyclase occurred at about 50 nM free Ca^{2+} ions. At free Ca^{2+} concentrations higher than about 200 nM no Mg^{2+}-dependent guanylate cyclase activity could be detected (Fig. 3).

When lysates were prepared in the presence of excess $CaCl_2$ (100 μM), and, subsequently, 15 s before the guanylate cyclase assay excess EGTA (5.6 mM) was added to reduce the free Ca^{2+} concentration, the inhibition of the enzyme by Ca^{2+} ions was partially (about 30%) reversed. This observation demonstrates that the inhibition of the enzyme by Ca^{2+} ions was not solely the result of some kind of irreversible inactivation, *e.g.* a Ca^{2+}-stimulated proteolytic breakdown of the enzyme protein. Omission of the divalent cation-chelator EGTA from

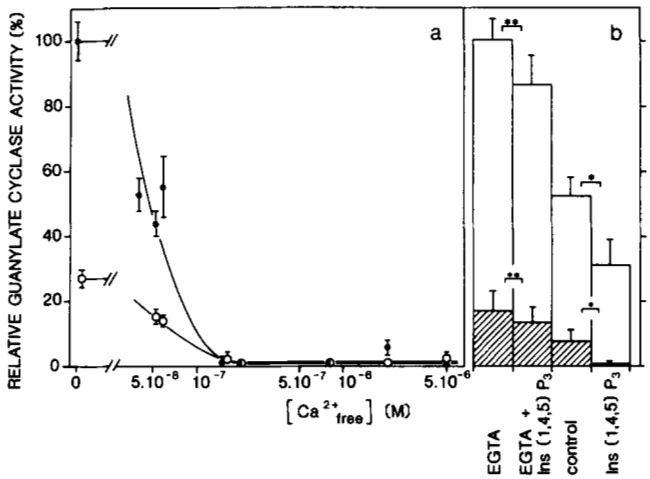

FIG. 3. **Effect of Ca^{2+} ions and inositol 1,4,5-trisphosphate on basal and GTPγS-stimulated, Mg^{2+}-dependent guanylate cyclase.** *D. discoideum* cell homogenates were prepared in lysis buffer in the absence or presence of 25 μM GTPγS (*open symbols* or *hatched bars*, and *filled symbols* or *open bars*, respectively). In *a*, the free Ca^{2+} concentration during lysis was varied by adding 1–6 mM $CaCl_2$ to lysis buffer and 5.9 mM EGTA. [Ca^{2+} free] = 0 M denotes the condition in which 5.9 mM EGTA was present and $CaCl_2$ was omitted. The K_D for the Ca + EGTA ⇌ Ca-EGTA equilibrium at pH 7.0 was taken 1.85×10^{-7} M (20). The free Ca^{2+} concentrations were calculated using: log[Ca^{2+}-free] = log K_D + log ([Ca-EGTA]/[EGTA-free]). Data are the means ± S.E. from 3 separate experiments. *b*, guanylate cyclase activity in homogenates prepared in the absence or presence of 5 mM EGTA or 10 μM inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), as indicated. Data are the mean ± S.E. of 2–7 lysates. *, the difference is significant at $p < 0.1$; **, the difference is not significant at $p > 0.4$ (Student's *t* test, non-paired).

lysis buffer resulted in about 50% lower guanylate cyclase activity than in the presence of EGTA, both in the presence and absence of GTPγS (11; and data not shown). Considering the strong inhibition of Mg^{2+}-dependent guanylate cyclase by micromolar concentrations of Ca^{2+} ions, and the specificity of EGTA for Ca^{2+} ions (20), the higher activity in the presence of EGTA can probably be attributed to the elimination from the homogenate of free Ca^{2+} ions originating from cells.

Ins(1,4,5)P_3, like Ca^{2+} ions, did not stimulate basal or GTPγS-stimulated guanylate cyclase (Fig. 3*b*). Rather, in the absence of EGTA, inhibition of guanylate cyclase was observed (significance $p < 0.1$). This may be explained by an Ins(1,4,5)P_3-mediated release of Ca^{2+} ions from intracellular stores present in the homogenates. Such a release has been demonstrated in permeabilized *D. discoideum* cells (21). Considering the sensitivity of Mg^{2+}-dependent guanylate cyclase to Ca^{2+} ions (Fig. 3*a*), the Ca^{2+} release induced by Ins(1,4,5)P_3 would be predicted to be about 40 nM. The above explanation agrees with the observation that Ins(1,4,5)P_3 did not inhibit guanylate cyclase in the presence of EGTA (Fig. 3*b*).

Ca^{2+} ions (2 or 100 μM) also strongly inhibited Mg^{2+}-dependent guanylate cyclase in homogenates, prepared from cells that had been treated with saponin (1 mg/ml, 30 min). The Mg^{2+}-dependent enzyme was also inhibited by Ca^{2+} ions when saponin (1 mg/ml) was added to homogenates immediately after cell lysis and prior to the guanylate cyclase assay (data not shown). Thus, the difference in the effects of Ca^{2+} on cGMP production in permeabilized cells (3, 4) and in cell homogenates does not result from the use of saponin in the former system. When we tried to reproduce the previous findings with saponin-permeabilized cells (3, 4), we were able to induce a slight cGMP accumulation using Ins(1,4,5)P_3 (maximally 10–25% of the accumulation induced by chemoattractants); however, we were unable to induce a cGMP response by use of Ca^{2+} ions.[2]

Effect of pH and Adenine Nucleotides—Guanylate cyclase in homogenates prepared both in the absence and presence of GTPγS had optimal activity between pH 7.5 and 8.0 (not shown). This is the same pH optimum as reported for Mn^{2+}-dependent guanylate cyclase in *Dictyostelium* (10). Below pH 7.0 almost no cyclase activity was detectable. Above pH 7.0 the cyclase activity in homogenates not containing GTPγS was also less than about 50% of the activity in the presence of GTPγS. Hence, no evidence was found for pH-dependent uncoupling of guanylate cyclase from its GTPγS sensitivity.

The effect of adenine nucleotides on the GTPγS-stimulated guanylate cyclase was the same as the effect on the enzyme in the absence of GTPγS, described before (11). Thus, App(NH)p stimulated guanylate cyclase in the presence of GTPγS the same amount (2–3-fold) as in its absence, whereas ATP and ATPγS had no effect on the basal or GTPγS-stimulated enzyme activity (11, and data not shown). As noted before (11), the latter observation suggests that the stimulation of the enzyme by guanine nucleotides (see below) is not mechanistically related to that by adenine nucleotide(s).

Characterization of the Effect of Guanine Nucleotides on Mg^{2+}-dependent Guanylate Cyclase

Dose response curves of the effect of guanine nucleotides on the activity of Mg^{2+}-dependent guanylate cyclase are shown in Fig. 4. The most potent guanine nucleotide was GTPγS, which stimulated half-maximally at about 1 μM. Maximal, about 4-fold, stimulation was reached at about 10^{-4} M GTPγS. A similar -fold amount of stimulation was observed

[2] P. J. M. Van Haastert and R. J. W. DeWit, unpublished observations.

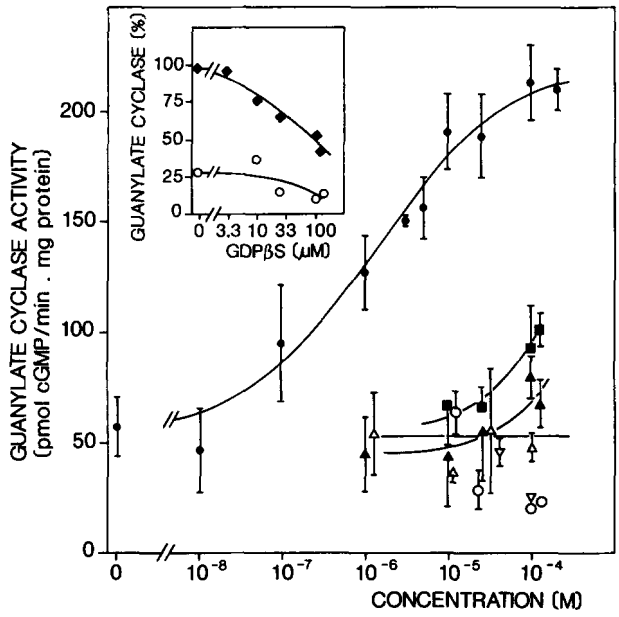

FIG. 4. **Dose-response curves of the effect of guanine nucleotides on Mg^{2+}-dependent guanylate cyclase.** Homogenates were prepared in the presence of various guanine nucleotides, and the enzyme activities were measured as described in the footnote to Table I. ▲, GTP; ●, GTPγS; ■, Gpp(NH)p; △, GDP; GDPβS; ▽, GMP. The *inset* shows the guanylate cyclase activity in homogenates, prepared in the presence (●) or absence (○) of 3.3 μM GTPγS and various concentrations of GDPβS as indicated. The figure is a combination of 5 experiments; presented are the means ± S.E.

with Mg^{2+}-dependent guanylate cyclase from vegetative and aggregation-competent *Dictyostelium* cells. The GTP analogue Gpp(NH)p stimulated guanylate cyclase up to about 2-fold at concentrations around 10^{-4} M. Inclusion of GTP at concentrations of about 10^{-4} M during cell lysis also resulted in a small increase in the cGMP production by homogenates (Fig. 4). This effect, however, may be explained by an increase in the substrate concentration for guanylate cyclase, taking into account the K_M of the enzyme for GTP (see below) and the subsaturating GTP concentration normally used in the enzyme reactions. Therefore, in some experiments, the GTP concentration in the reaction mixtures was adjusted so that homogenates prepared in the presence of GTP were assayed at the same GTP concentration as controls from homogenates that had not received GTP during lysis. In these experiments the presence of 4 mM GTP during cell lysis resulted in only 40% more cGMP synthesis as compared to the synthesis by lysates prepared in the absence of GTP.

Guanosine diphosphates, like GDP and GDPβS, and guanosine monophosphate (GMP) did not activate guanylate cyclase (Fig. 4). Rather, they tended to inhibit the enzyme. GDPβS also antagonized the activation by GTPγS, when present in excess of this compound (Fig. 4, *inset*). This might be explained by competition of GDPβS with GTPγS for a regulatory site or by competition of GDPβS with GTP or GTPγS at the catalytic site of the enzyme. The latter explanation is favored by the observation that GDPβS inhibited the cGMP synthesis in the presence and absence of GTPγS to relatively the same amount (about 50% at 100 μM GDPβS, Fig. 4, *inset*).

The activation of *Dictyostelium* guanylate cyclase by guanosine triphosphates has some characteristics similar to the activation of *Dictyostelium* adenylate cyclase, with regard to its nucleotide specificity and the property to give highest activity shortly after cell lysis (13). A difference, however, is

that guanylate cyclase activity decreased rather than increased during incubation at 0 °C in the presence of GTPγS (Figs. 1 and 2), while for adenylate cyclase such an incubation is required to obtain maximal activity (13). Another difference between both enzymes was observed when studying the aggregation-less mutant *synag 7* (also called N7). Activation of membrane-bound adenylate cyclase requires the presence of a soluble cytosolic factor and does not occur in this mutant, which has a defect in this factor (13, 22). As shown in Table II, homogenates of mutant *synag 7* contained about 5-fold less guanylate cyclase activity than wild type homogenates, as measured with Mn^{2+}-GTP as a substrate. Basal Mg^{2+}-dependent guanylate cyclase activity could not be detected. However, when GTPγS was included in homogenates a Mg^{2+}-dependent guanylate cyclase activity, comparable to the activity in the presence of Mn^{2+} ions, was apparent. Thus, in contrast to adenylate cyclase in mutant *synag 7* (13, 22), guanylate cyclase showed no defect in its activation by GTPγS (Table II). These observations suggest that, despite some superficial resemblances, *Dictyostelium* guanylate cyclase and adenylate cyclase are activated by guanine nucleotides by different mechanisms.

Kinetics of GTPγS-stimulated, Mg^{2+}-dependent Guanylate Cyclase

The kinetics of Mg^{2+}-dependent guanylate cyclase were studied under three different conditions. Either homogenates were prepared in the absence of exogenously added guanine nucleotides and the activity of the enzyme was measured with various concentrations of GTP or GTPγS, or homogenates were prepared in the presence of a low concentration of GTPγS and the enzyme activity was determined with various concentrations of GTP during the reaction. These experiments might shed light on the mechanism of activation of the enzyme by GTPγS and answer the question whether GTPγS was (also) a substrate for the enzyme.

The results of these kinetic measurements are compiled in Table III. Under each condition tested, the behavior of guanylate cyclase conformed to Michaelis-Menten kinetics. Relatively, the lowest activity was detected with the enzyme prepared in the absence of GTPγS, using GTP as substrate (Table III, part I). Guanylate cyclase also effectively used GTPγS as a substrate, showing the same V_{max} and about a 3-fold lower K_M with GTPγS than with GTP (Table III, part II). When GTPγS was present during cell lysis and the enzyme activity was subsequently measured with GTP the highest activity was detected, resulting from both a high V_{max} and a low K_M under this condition (Table III, part III).

These results suggest that relatively low GTPγS concentrations (1 μM) affect guanylate cyclase in two ways, both leading to increased cGMP synthesis in homogenates with GTP. Firstly, GTPγS lowers the K_M of the enzyme for GTP. Secondly, GTPγS increases the V_{max} of guanylate cyclase for GTP. At relatively high concentrations (≈100 μM) GTPγS also becomes a substrate for the enzyme.

GTPγS Stimulates Particulate Guanylate Cyclase

Mn^{2+}-dependent guanylate cyclase in *Dictyostelium* has been found in both soluble and particulate cell fractions (7, 9,

TABLE II

Activation of Mg^{2+}-dependent guanylate cyclase by GTPγS in cell-free preparations of Dictyostelium mutant synag 7 and wild type NC4

Lysis and reactions were as described in Table I. GTPγS, if indicated, was present at 25 μM; when $MnCl_2$ (5 mM) was present in the reaction mixture, the GTP concentration was 2 mM, instead of 0.3 mM, as under standard conditions. *Synag 7* cells were grown and developed until aggregation competence as described before (22).

Strain	Additions		Guanylate cyclase	
	During lysis	During reaction	*pmol/min·mg protein ± S.E. (n)*	
Synag 7			2 ± 2	(4)
Synag 7	GTPγS		40 ± 16	(4)
Synag 7		$MnCl_2$ (GTP)	35 ± 7	(3)
NC4			56 ± 10	(9)
NC4	GTPγS		188 ± 13	(7)
NC4		$MnCl_2$ (GTP)	135 ± 16	(6)

TABLE III

Kinetic parameters of Mg^{2+}-dependent guanylate cyclase in D. discoideum homogenates under various conditions

The kinetic parameters are the mean ± S.E. from 3–6 experiments. Homogenates were prepared in the absence or presence of GTPγS, kept at 0 °C, and 90 s after cell lysis the guanylate cyclase activity was measured in reaction mixtures containing 0.05–2 mM GTP or 0.0125–0.9 mM GTPγS. In all cases analyzed the data showed good fits to linear Michaelis-Menten kinetics. 1 mg of protein is equivalent to 3.8×10^7 cells.

Preparation	Compound varied in reaction mixture	K_M	V_{max}
		μM	*pmol cGMP/min·mg protein*
I. Lysate prepared in the absence of GTPγS	GTP	341 ± 66	123 ± 8
II. Lysate prepared in the absence of GTPγS	GTPγS	116 ± 19	115 ± 18
III. Lysate prepared in the presence of 25 μM GTPγS	GTP	87 ± 7	252 ± 78

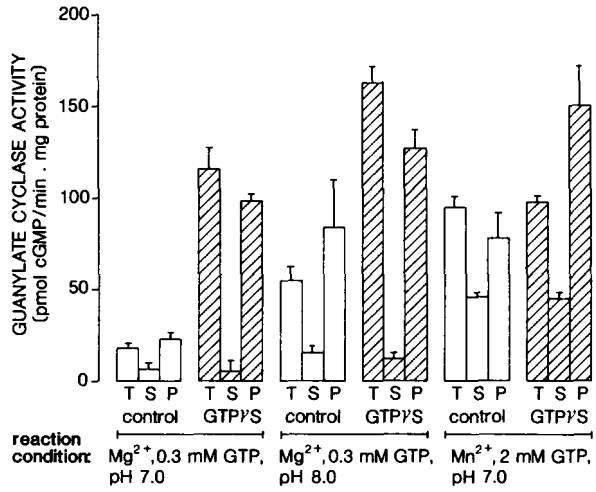

FIG. 5. **Subcellular distribution of basal and GTPγS-stimulated, Mg^{2+}-dependent guanylate cyclase.** Cell homogenates were prepared in the absence or presence of 25 μM GTPγS and centrifuged for 1 min at 10,000 × *g* (4 °C), (*open* and *hatched bars*, respectively). Supernatants were collected; pellets were resuspended without dilution in lysis buffers plus or minus 25 μM GTPγS in the cases where the homogenate contained, or did not contain, GTPγS, respectively. The guanylate cyclase activity in each of the fractions was assayed in duplicate, at about 4 and 7 min after cell lysis, in reaction mixtures containing GTP, Mg^{2+}, and Mn^{2+} ions and having a pH as indicated. *T*, homogenate; *S*, supernatant; *P*, pellet. The experiment was reproduced two times (assays with Mg^{2+}-GTP at pH 7.0) or once (others). The *error bars* represent the mean standard deviations of the measurements.

10, 16). To investigate which of these enzyme forms corresponded to the Mg^{2+}-dependent enzyme and which form was activated by GTPγS, cell homogenates were made in the presence or absence of GTPγS, rapidly centrifuged, and the cyclase activity in sediment and supernatant was measured with Mg^{2+}-GTP at pH 7.0. Measurements were also done with Mg^{2+} ions at pH 8.0 and with Mn^{2+}/2 mM GTP, to investigate whether the enzyme activity observed under these conditions behaved differently from the activity detected with Mg^{2+}-GTP at pH 7.0.

Mg^{2+}-dependent guanylate cyclase was found both in pellet and supernatant fractions after 1 min centrifugation at 10,000 × *g* (Fig. 5). When homogenates prepared in the presence of GTPγS were fractionated, increased guanylate cyclase activity was measured only in 10,000 × *g* pellets, not in supernatants (Fig. 5). The same results were obtained when the enzyme was measured with Mg^{2+} ions at pH 8.0 and with Mn^{2+}/2 mM GTP. No evidence was found, using these assay conditions, for a guanylate cyclase other than the Mg^{2+}-dependent enzyme and behaving differently from the latter enzyme. Only, measuring at pH 8.0 or with Mn^{2+}/2 mM GTP, the activity in preparations not containing GTPγS was higher than when measured with Mg^{2+}-GTP at pH 7.0 (Fig. 5). This apparently results from a higher intrinsic activity of Mg^{2+}-dependent guanylate cyclase.

DISCUSSION

In this paper we report on some of the characteristics of a highly active Mg^{2+}-dependent guanylate cyclase in *D. discoideum* membranes. This enzyme may be identical to the enzyme regulated *in vivo* via cell surface receptors, as we suggested before (11). The enzyme is stimulated *in vitro* by the GTP analogues GTPγS and Gpp(NH)p, the ATP analogue App(NH)p, and inhibited by nanomolar concentrations of Ca^{2+} ions. These effects may reflect regulatory mechanisms that operate *in vivo*, especially because they were established under *in vitro* conditions that are more physiologic than were used previously, *i.e.* using Mg^{2+}-GTP instead of Mn^{2+}-GTP as a substrate for the enzyme. Optimal enzyme activity was obtained in homogenates, immediately after lysis of cells in the presence of GTPγS, App(NH)p, and the Ca^{2+} chelator EGTA. Under these conditions a V_{max} = 66 pmol/min·10^7 cells was estimated with Mg^{2+}-GTP as substrate. This activity is close to the activity estimated for guanylate cyclase *in vivo* that is stimulated via cell surface receptors (60–90 pmol/min·10^7 cells; 12, 19, 23).

Several mechanisms can be envisaged to explain the higher activity of Mg^{2+}-dependent guanylate cyclase in the presence of GTPγS: 1) GTPγS may be a substrate for the enzyme; 2) GTPγS may be stabilizing the enzyme; and 3) GTPγS may be an allosteric activator. There is evidence for each of these mechanisms.

(*a*) That GTPγS might be a substrate is strongly suggested by the observation of GTPγS-dependent cGMP synthesis, characterized by a V_{max} similar to the V_{max} with GTP (Table III). This GTPγS-dependent synthesis was obtained when GTPγS was the sole (exogenous) guanosine triphosphate in the reaction.

(*b*) In favor of the idea that GTPγS stabilizes the enzyme is the observation that the presence of GTPγS during cell lysis resulted in higher cGMP synthesis than presentation of GTPγS only at the time of the reaction (Fig. 2). Furthermore, the enzyme activity decayed more rapidly in the absence than in the presence of GTPγS (Fig. 1).

(*c*) Suggesting allosteric activation of guanylate cyclase by GTPγS, finally, is the observation that in the presence of GTPγS the enzyme has a significantly lower K_M for substrate than in its absence (Table III). This lower K_M was observed when GTPγS was used as the sole substrate for the enzyme, as well as with GTP as a substrate in the presence of a subsaturating concentration of GTPγS (Table III, part III). The kinetic data rule out the possibility that the higher activity of the enzyme under the latter conditions was only because GTPγS was a substrate in addition to GTP.

We propose the following explanation for the effect of GTPγS on the activity of Mg^{2+}-dependent guanylate cyclase in homogenates. In addition to the catalytic site there is an allosteric site on the enzyme with high affinity for guanine nucleotides. This allosteric site may be located on the enzyme itself or on a separate component, *e.g.* a G-protein (24). Occupation of the allosteric site by GTPγS lowers the K_M of the enzyme and in addition leads to its stabilization. The latter effect is observed as an increase in the V_{max} after some time of incubation with GTPγS, for instance of the labile enzyme that is present in homogenates immediately after cell lysis. GTPγS is also a substrate of the enzyme and therefore does not competitively inhibit the cGMP synthesis when GTP is present as substrate. GTP apparently has a much lower affinity for the allosteric site than GTPγS. The affinity of the enzyme for the natural substrate GTP is increased by the presence of GTPγS because this nucleotide occupies the allosteric site more permanently than GTP. Whether this occurs by a mechanism analogous to G-proteins (24) remains to be investigated. As yet, we have no direct evidence for the involvement of G-proteins in the regulation of Mg^{2+}-dependent guanylate cyclase; the properties of the enzyme were not found to be altered by treatment of cells with cholera or pertussis toxin (data not shown).

The role of Ca^{2+} in the regulation of guanylate cyclase appears to be contrary to the role of guanine nucleotides, *i.e.* inhibitory. It appears that the Mg^{2+}-dependent guanylate cyclase is much more sensitive to Ca^{2+} than the Mn^{2+}-dependent enzyme, as Mn^{2+}-dependent guanylate cyclase is only weakly inhibited by Ca^{2+} ions (9).[3] Micromolar concentrations of Ca^{2+} ions seem to be released in the cytoplasm of *D. discoideum* cells in response to receptor-induced activation of a phosphatidylinositol cycle (21). The released micromolar concentrations of Ca^{2+} ions would be sufficient for a complete arrest of the enzyme, as is suggested by the sensitivity of the Mg^{2+}-dependent enzyme for Ca^{2+} ions. Thus, a basal Ca^{2+} concentration in the cytoplasm of 100 nM or more might keep guanylate cyclase in an inactive state. An interesting possibility is that receptor-mediated stimulation of guanylate cyclase *in vivo* is brought about by a temporal relaxation of a tonic inhibition of the enzyme by Ca^{2+} ions.

The effects of Ca^{2+} ions and Ins(1,4,5)P_3 we observed on cGMP synthesis *in vitro* are opposite to those reported by Europe-Finner *et al.* (3, 4) with permeabilized *D. discoideum* cells. In contrast to these authors, we only observed inhibitory effects of Ca^{2+} ions on guanylate cyclase. We were unable to reproduce their results with Ca^{2+} ions in permeabilized cells, while we found only very weak responses with Ins(1,4,5)P_3. We have at present no explanation for the apparently differing findings. The present results are a first characterization of a guanylate cyclase from *D. discoideum* that can be assayed *in vitro* with a substrate, probably identical to the physiological substrate, Mg^{2+}-GTP. They should provide a basis for further *in vitro* studies on the regulation of the enzyme.

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[3] P. M. W. Janssens, C. C. C. De Jong, A. A. Vink, and P. J. M. Van Haastert, unpublished observations.

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